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Distinct isocomplexes of the TRAPP trafficking factor coexist inside human cells

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Abstract **The transport protein particle (TRAPP) complex is required for proper vesicular transport from the ER to the Golgi. The composition of yeast TRAPP is well characterized, but the organization of mammalian TRAPP complex remains elusive. Using a tandem affinity purification (TAP) approach, we provide first experimental proof for the association of NIBP (NIK/IKKβ binding protein) with Bet3 and find two human paralogs of Trs33 (A and B) associated with Bet3. Interaction studies and gel filtration analysis reveal that both proteins are part of human TRAPP and might mark two distinct isocomplexes that exert different functions in the regulation of ER-to-Golgi traffic.**

Structured summary:
MINT-6784845:
Bet3 (uniprotkb:O43617) *physically interacts* (MI:0218) with *Trs33B* (uniprotkb:Q86SZ2) by *anti bait coimmunoprecipitation* (MI:0006)
MINT-6785053:
Trs33B (uniprotkb:Q86SZ2) *physically interacts* (MI:0218) with *Bet3* (uniprotkb:O43617) and *Sedl* (uniprotkb:O14582) by *anti bait coimmunoprecipitation* (MI:0006)
MINT-6784856:
Bet3 (uniprotkb:O43617) *physically interacts* (MI:0218) with *Trs33A2* (uniprotkb:O75865-2) by *anti bait coimmunoprecipitation* (MI:0006)
MINT-6785038:
Trs33A1 (uniprotkb:O75865-2) *physically interacts* (MI:0218) with *Sedl* (uniprotkb:O14582) and *Bet3* (uniprotkb:O43617) by *anti bait coimmunoprecipitation* (MI:0006)
MINT-6784879:
Bet3 (uniprotkb:O43617) *physically interacts* (MI:0218) with *NIBP* (uniprotkb:Q96Q05) by *tandem affinity purification* (MI:0676)
MINT-6785068:
Trs33B (uniprotkb:Q86SZ2), *Trs33A2* (uniprotkb:O75865-2) and *Bet3* (uniprotkb:O43617) *colocalize* (MI:0403) by *molecular sieving* (MI:0071)
MINT-6785415:
Bet3 (uniprotkb:O43617) *physically interacts* (MI:0218) with *Trs33A1* (uniprotkb:O75865) by *anti bait coimmunoprecipitation* (MI:0006)

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1. Introduction

Vesicular transport in eukaryotic cells follows a modular organization, where different trafficking pathways are mediated and regulated by functionally related submodular entities [1]. A number of regulatory components that are required after vesicle scission and prior to docking and membrane fusion have been grouped in the class of tethering factors [2]. They cooperate with coat proteins, SNAREs and Rab GTPases, acting upstream or downstream of Rabs [3]. Transport protein particle (TRAPP) is implicated in transport from ER to the Golgi and present in two isoforms in yeast [4]. The seven-subunit TRAPP I complex is involved in the recruitment of ER-derived vesicles to the *cis*-Golgi and binds to COPII [4,5]. TRAPP II contains three additional subunits and is located at the *trans*-Golgi, where it is required for retrograde transport from endosomes [4,6]. Both complexes differ in localization, with TRAPP I co-fractionating with early Golgi compartments, but TRAPP II locating to the late Golgi [6–8], and have varying specificity as guanine nucleotide exchange factors (GEF) for the Rab GTPases Ypt1p and Ypt31/32p, respectively [9–12]. Only one TRAPP complex could be identified according to its size in mammalian cells [13] which functions in the formation of vesicular tubular clusters (VTCs), mammalian pre-Golgi compartments [14].

Structural studies revealed that two protein families (Bet3 and Bet5 family) exist within the complex with three members each [15,16]. In yeast, these subunits assemble into one heptameric subcomplex with two copies of Bet3p and one copy of the remaining subunits [11]. The mammalian subunits, in contrast, form a trimeric (Bet3:Trs31:Sedl) and a tetrameric (Bet3:Trs33:Bet5:Trs23) subcomplex that do not stably associate in vitro [16], indicating that at least one additional subunit is required for the assembly of mammalian TRAPP. Two human paralogs can be identified for the TRAPP subunits Bet3, Sedl and Trs33. For Trs33 both variants are expressed in mice [17] and are interchangeable in their ability to form a tetrameric TRAPP subcomplex in vitro [16,18]. Furthermore, a *trs33a* knockout in mice [19] and mutations in the human

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SEDL gene [20] show only relatively mild phenotypes. Hence, different paralogs might mark distinct isocomplexes with specific or redundant functions in trafficking.

2. Materials and methods

2.1. TAP cell lines

Genes were cloned into pcDNA3 (Invitrogen) with an N-terminal TAP-Tag (NTAP). Stable HEK 293 cell lines were generated by geneticin selection. NTAP-Bet3 was purified according to [21]. Samples were analyzed by SDS–PAGE and mass spectrometry. Alternatively, pull-downs from NTAP cell lysates were performed with IgG (immunoglobulin G)-Sepharose (GE Healthcare) without further purification, allowing to detect NTAP-Bet3 using α-ProteinA antibody (Sigma).

2.2. Gel filtration

Recombinant Bet3:Trs33B and Bet3:Trs33B:Bet5:Trs23 complexes and Trs33 were expressed and purified essentially as described before [15,17,18]. Cleared HEK 293 cell lysate (150 mM NaCl, 50 mM Hepes, pH 7.5, 0.2% NP-40) was loaded on a Superdex 200 10/300 (Amersham) column. 1 ml fractions were collected and subjected to deoxycholate (DOC, 0.05%)/trichloracetic acid (TCA, 1.2%) precipitation. Samples were analyzed by SDS–PAGE and Western blotting using antibodies against Bet3, Trs33A and Trs33B. Recombinant TRAPP subcomplexes were loaded on the gel filtration column equilibrated with PBS containing 1 mM DTT.

2.3. Antibodies and siRNA

Affinity-purified antibodies specific for Trs33A and Trs33B were raised against the synthetic peptides CELWAMDPDPGPGGQK and CGVYKSAEQGEVENG, respectively, derived from the α1-α2 loop region of Trs33 (Biogenes/Biosyntan, Berlin). Antisera against Bet3 and Sedl were a kind gift from Dr. De Matteis. ProtA antibody was purchased from Sigma. Three siRNAs for each target were ordered from Eurogentec (see Supplementary information for sequences). siRNA transfection of HEK 293 was performed with Profectin 85 (Atugen, Berlin) at 100 nM final concentration. Cells were analyzed 72 h after transfection.

3. Results

3.1. Association of Trs33A, Trs33B and NIBP with Bet3

When searching databases, we identified three Trs33 paralogs expressed in man (Fig. 1A). Trs33B (UniProt:Q86SZ2) and Trs33A1 (UniProt:Q75865-1) share 56% identical and 72% similar residues. The splice isoform Trs33A2 (UniProt:Q75865-2) contains 14 additional amino acids inserted into the loop connecting helices α1 and α2, which is the least conserved region of the proteins.

We raised antibodies against Trs33A and Trs33B using peptides derived from the α1–α2 loops of the proteins (Fig. 1A). Testing the antibodies on recombinant protein showed that they specifically recognize the respective isoform without cross-reactivity (Fig. 1B). To examine expression of the isoforms in human cells we transfected HEK 293 with three siRNAs directed against each paralog. Western blot analysis revealed efficient knock-down of Trs33B and two Trs33A gene products (Fig. 1C). This clearly demonstrates that both splice variants, Trs33A1 and Trs33A2, and the paralog Trs33B are simultaneously expressed in mammalian cells.

To test whether Trs33 isoforms interact with Bet3 inside cells we used a stable NTAP-Bet3 cell line for pull-down experiments (Fig. 2A). Western blot analysis of NTAP-Bet3 precipitations using Trs33 isoform-specific antibodies revealed that

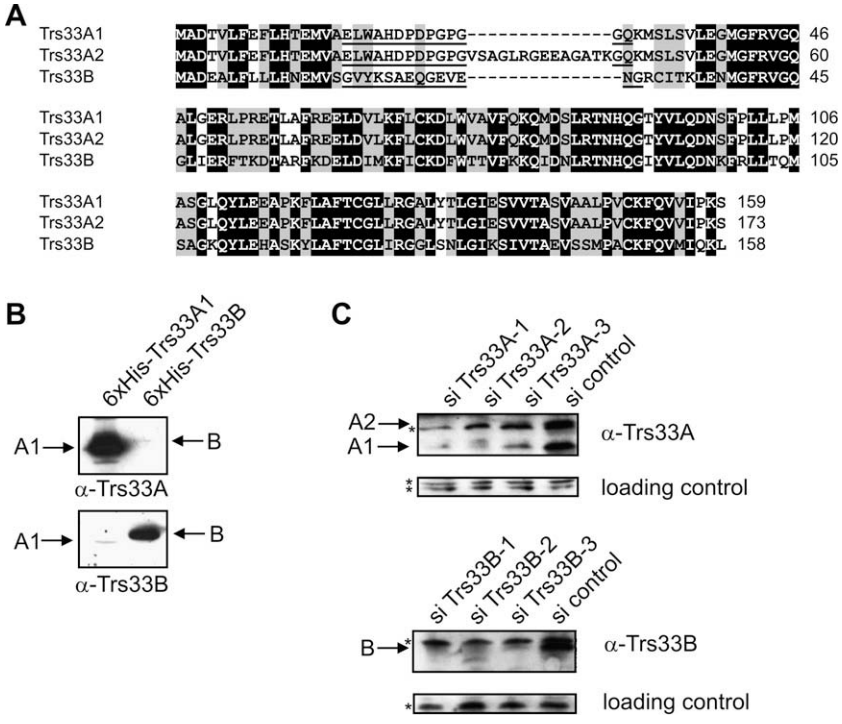

Fig. 1. (A) Sequence alignment of Trs33A1, Trs33A2 and Trs33B. Identical residues are shaded black, similar residues gray. The peptide regions targeted for antibody generation are underlined. (B) Antibodies against Trs33A and Trs33B were tested on lysates of *Escherichia coli* expressing recombinant 6xHis-Trs33A1 and 6xHis-Trs33B. (C) siRNA knock-down of Trs33A and Trs33B. Unspecific bands serve as loading control and are marked by asterisks.

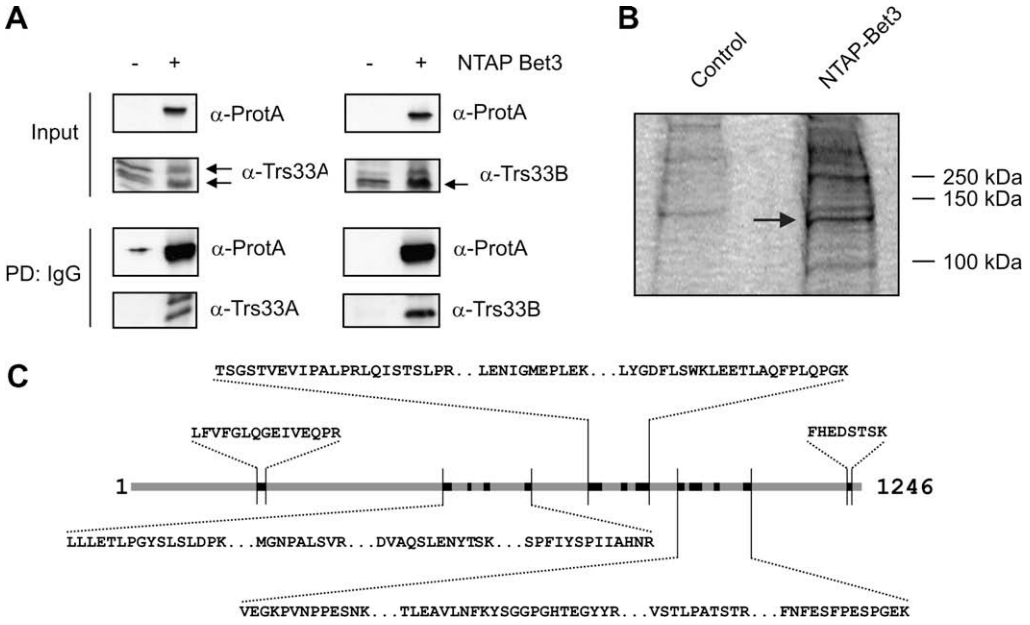

Fig. 2. (A) IgG-pull-downs from cell lines stably expressing NTAP-Bet3. Western blot analysis was performed with antibodies against ProtA, Trs33A and Trs33B. Both Trs33 isoforms are found to be associated with Bet3. (B) Tandem affinity purification from HEK 293 cells (control) and the NTAP-Bet3 cell line. Samples were loaded on a SDS–PAGE gel and stained with Coomassie. The protein identified as NIBP is marked by an arrow. (C) Positions of the peptide fragments that were characterized by mass spectrometry in the sequence of NIBP.

all isoforms, Trs33A1, Trs33A2 and Trs33B associate with Bet3 (Fig 2A).

We further used the NTAP-Bet3 cell line to investigate the composition of human TRAPP. The small subunits from the Bet3 and Bet5 protein families are well conserved between man and yeast, but the remaining large subunits are poorly described. Mammalian orthologs have been suggested based on sequence conservation for Trs120p [22] and interaction studies for Trs130p and Trs85p [23]. With the TAP approach we find several proteins that were specifically co-purified with NTAP-Bet3 (Fig. 2B). Using mass spectrometry analysis we identified one of the proteins that interact with Bet3 as NIBP (NIK/IKKβ binding protein, UniProt:Q6ZQT3, ~140 kDa), which shares 16% identical residues with Trs120p (Fig. 2C). For NIBP, a role in modulation of cytokine-induced NF-κB signaling has been proposed [24], but phylogenetic relationship of Trs120p and NIBP has also been described, suggesting a function within the human TRAPP complex [22]. Our finding provides the first experimental evidence that NIBP is a subunit of human TRAPP. This is further supported by our observation that NIBP can bind to Ehoc-1, another human TRAPP subunit (unpublished).

3.2. Trs33A and Trs33B are part of fully assembled TRAPP

Because the Bet3:Trs33 complexes represent a stable subassembly of TRAPP, incorporation of Trs33 isoforms in fully assembled TRAPP remains to be shown. Therefore, stable NTAP-Trs33A1 and NTAP-Trs33B cell lines were generated (Fig. 3A). In Trs33 pull-downs from these cells the interaction of both isoforms with Bet3 could be verified. In addition, Sedl was also found to be specifically associated with Trs33A and B. This interaction is probably indirect, as Trs33 and Sedl have been shown to be part of distinct mammalian TRAPP subassemblies that do not join without additional factors in vitro [16]. Thus, our data strongly suggest that both Trs33 paralogs are part of fully assembled TRAPP.

To address the question whether Trs33A and Trs33B can form TRAPP complexes independent of each other, Trs33 isoforms were knocked down separately in the NTAP-Bet3 cell line. Knock-down of one Trs33 isoform did not affect association of the remaining paralog with Bet3 (Fig. 3B). Equivalent results could also be observed with a different set of siRNAs (not shown). These observations reveal that Trs33 isoforms bind to Bet3 independently of each other. Reconstitution of both mammalian and yeast TRAPP complexes has demonstrated that two copies of Bet3, but only one copy of Trs33, can be incorporated into TRAPP [12,16]. Therefore, incorporation of different Trs33 isoforms into TRAPP results in different co-existing TRAPP complex subpopulations.

3.3. Trs33A and Trs33B are integral parts of endogenous TRAPP complexes

To investigate the composition of the endogenous mammalian TRAPP complex, lysates of HEK 293 cells were analyzed using gel filtration (Fig. 4A). Bet3 was found to be present in two distinct pools. The high-molecular-weight pool (fractions 3 and 4, ~600 kDa) is likely to represent the fully assembled TRAPP. In contrast, fractions 7 and 8 (~100–70 kDa) represent a pool of Bet3-containing lower molecular weight subassemblies of TRAPP. All three Trs33 isoforms show a similar distribution in gel filtration. Most importantly, Trs33A1, Trs33A2 and Trs33B co-elute with Bet3 in fraction 3, providing evidence that Trs33A and Trs33B are both part of intact cellular TRAPP complexes. However, only little Trs33A is found in fraction 4, and for the low-molecular-weight pool, Trs33A is distributed over fractions 7–9, whereas Trs33B migrates in fractions 8 and 9. For a more accurate size determination of the separated complex fractions, recombinant

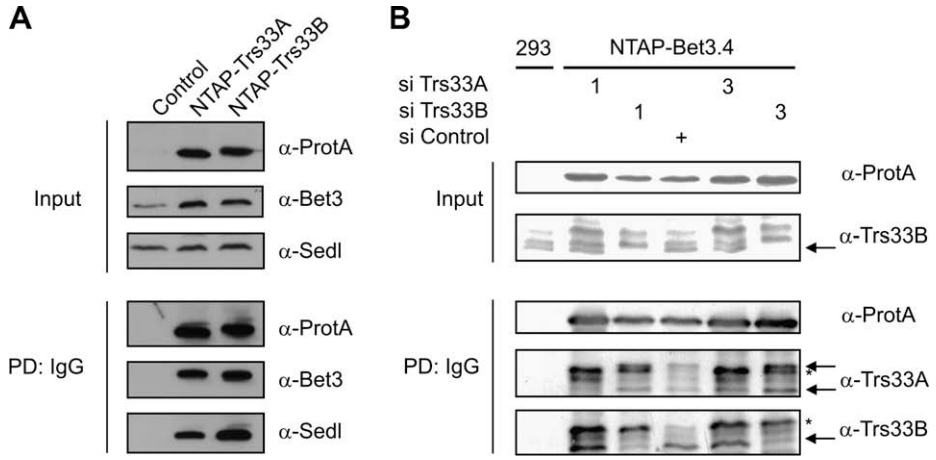

Fig. 3. (A) IgG-pull-downs from cell lines stably expressing NTAP-Trs33A1 or NTAP-Trs33B. Western blots were probed with antibodies against ProtA, Bet3 and SedI. Co-precipitation of Bet3 and SedI with Trs33A and Trs33B reveals that both isoforms are part of fully assembled TRAPP. (B) NTAP-Bet3 cells were transfected with control siRNA, or siRNAs targeting Trs33A or Trs33B. Cleared cell lysates were subjected to pull-down with IgG-Sepharose and Western blot analysis. The interaction of Trs33B with Bet3 is not affected by knock-down of Trs33A and vice versa. An unspecific band below Trs33A2 that is not present in the knock-down with control siRNA is marked by an asterisk. HEK 293 cells were used as control.

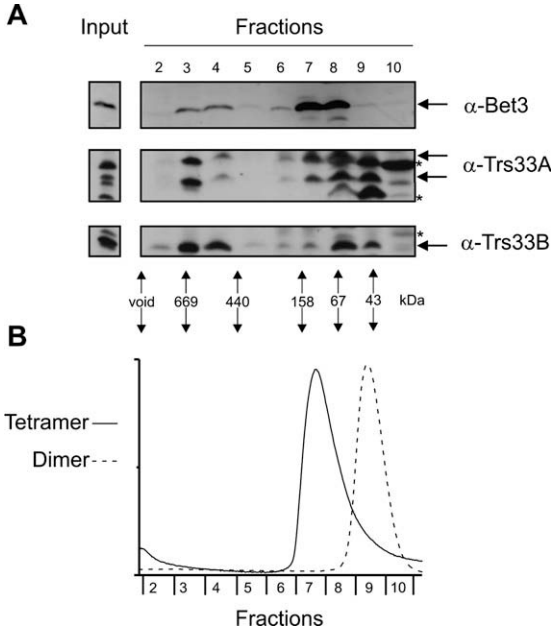

Fig. 4. Gel filtration of endogenous and recombinant TRAPP complexes. (A) Western blot analysis of gel filtration chromatography of HEK 293 cell lysate. Trs33A and Trs33B co-elute with Bet3 in a high- and a low-molecular-weight pool. Elution volumes of standard proteins are marked by arrows. (B) Elution profiles of recombinant Bet3:Trs33B dimer and Bet3:Trs33B:Bet5:Trs23 tetrameric subcomplex.

TRAPP subcomplexes were also analyzed by gel filtration (Fig. 4B). Comparison with the elution profile of endogenous Bet3 shows that the low-molecular-weight pool of Bet3 corresponds to the size of the tetrameric subcomplex (Bet3:Trs33B:Bet5:Trs23) rather than the Bet3:Trs33B heterodimer. This indicates that Bet3 is predominantly part of intact TRAPP or larger subassemblies, probably the tetrameric and trimeric TRAPP subcomplexes [16]. In contrast, Trs33 seems to be able to form (homo-)dimeric subassemblies. Considering the slightly different distributions of Trs33A and Trs33B at high and low molecular weight, the proteins might also assemble into complexes of similar size but variable composition and architecture. It remains to be investigated whether the low-molecular-weight pool of TRAPP subunits is of physiological significance or the result of complex disruption during gel filtration.

4. Discussion

Our data clearly show that at least three distinct isoforms of TRAPP marked by the incorporation of different Trs33 paralogs and splice variants coexist in human cells. From our gel filtration analysis we conclude that, whereas two TRAPP complexes are found in yeast, the architecture of the mammalian TRAPP complex does not suggest a comparable mechanism. Assembled human TRAPP presumably contains all TRAPP I and II-homologous subunits, including NIBP. Instead, the presence of different isoforms of some subunits of human TRAPP adds complexity to the regulation of mammalian vesicular transport compared to yeast. Unique functions for protein isoforms have been reported for the retromer subunit Vps26 [25]. Here, isoforms were found to distinctively localize to the plasma membrane or endosomes. Other examples for larger sets of isoforms utilized in the mammalian transport network are the ER-to-Golgi Rab GTPase Rab1, subunits of the exocyst and class C Vps tethering complexes [2], and the COP II adaptor proteins Sec23/24 and Sar1 [26]. It is tempting to speculate that the different variants of these components regulate the transport of distinct subsets of cargo or vesicles between similar compartments. Hence, it is important to identify the roles of these trafficking factor isoforms for a better understanding of vesicular transport regulation.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2008.09.056.

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